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A β oligomerization is associated with the generation of a typical peptide fragment fingerprint.

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Conflict of interest: none

ABSTRACT

Amyloid-beta ($A\beta$) peptide oligomerization plays a central role in the pathogenesis of Alzheimer's disease (AD) and $A\beta$ oligomers are collectively considered an appealing therapeutic target for the treatment of AD. However, the molecular mechanisms leading to the pathological accumulation of oligomers are unclear and the exact structural composition of oligomers is being debated. Using targeted and quantitative mass spectrometry, we reveal site-specific $A\beta$ autocleavage during the early phase of aggregation, producing a typical $A\beta$ fragment signature and that truncated $A\beta$ peptides can form stable oligomeric complexes with full-length $A\beta$ peptide. We show that the use of novel anti- $A\beta$ antibodies raised against these truncated $A\beta$ isoforms allows for monitoring and targeting the accumulation of truncated $A\beta$ fragments. Antibody-enabled screening of transgenic models of AD, as well as human post-mortem brain tissue and cerebrospinal fluid revealed that, aggregation-associated $A\beta$ cleavage is a highly relevant clinical feature of AD.

1. INTRODUCTION

Alzheimer disease (AD) is a progressive neurodegenerative disorder that is manifested as a gradual decline in memory and cognitive function. A number of studies indicate that soluble oligomers might account for the AD-associated decline in synaptic plasticity [1, 2] and that inhibition of natural A β oligomerization rescues deficits in long-term potentiation (LTP) [3]. Several types of A β assemblies of dimeric and trimeric [4] [1] [2] or dodecameric (A β * 56) [5] nature have been observed *in vitro* and *in vivo* in transgenic mouse models, human cerebrospinal fluid (CSF) [6], and post-mortem AD brain extracts [7] [8], with the higher molecular weight species being considered the main neurotoxic culprit associated with cognitive dysfunction. Collectively A β oligomers can be considered as an appealing diagnostic and therapeutic target. However, the general morphological heterogeneity and, to some extent, metastable structure renders an antibody based targeting and detection of oligomers difficult. Therefore, the development of specific anti-oligomeric based therapeutics remains challenging.

Cerebrospinal fluid (CSF) analyses from AD patients indicate that the presence of A β oligomers correlates with a concomitant decrease in A β 42 levels. CSF levels of total and phosphorylated Tau protein [9], tissue transglutaminase (tTGase) [10], ubiquitin [11], A β oligomers [12] as well as changes in A β 1-42 concentration, together with the presence of particular A β truncations [13] have been collectively suggested as useful biomarkers in AD.

Previously, mass spectrometry (MS) based analysis of CSF revealed a specific A β peptide fragment signature in sporadic AD patients [14-16] and it has been reported that truncated A β is known to represent more than 60% of all A β species found in non-demented as well as in AD individuals [17]. These findings may suggest that A β oligomers could consist of a heterogeneous morphological entity of full-length A β 40 and A β 42 as well as truncated A β

isoforms, of which the latter may serve as an important molecular seed during peptide aggregation [18]. Similarly, a recent report showed that the aqueous phase of human AD brain extracts contained SDS-stable A β species of a molecular weight range of 6-7kDa and that these A β species may form part of larger A β aggregates [19].

In this work we sought to identify a “molecular crosstalk” during the lag phase of A β peptide aggregation that typically precedes the pathological accumulation of neurotoxic oligomers. Here, we have identified site specific autocleavage of A β peptide and report a typical peptide fragment fingerprint, which may be associated with the early nucleation process of A β aggregation. Using targeted and quantitative MS, we reveal a highly reproducible A β fragment signature with a significant abundance of C-terminal peptide amidation. Moreover, we show that these truncated A β peptides have a particularly high propensity in forming SDS-stable low molecular weight oligomers of dimeric and trimeric nature. These findings have enabled us to develop novel neo-epitope antibodies that selectively bind to the gradual accumulation of truncated A β isoforms during the early phase of peptide aggregation. Our targeted analysis of human brain tissue extracts and CSF revealed that A β cleavage within the peptide’s β -turn region is a highly relevant feature observed in AD.

2. METHODS

2.1 A β peptide preparation.

Full-length wild type (wt) A β peptides A β 1-40, arctic mutant A β 1-40 (Arc) and A β 1-42 A β (Dr. James I. Elliott, Yale University, USA) were dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) at a concentration of 1mg/ml, followed by a 10-min sonication to break any preformed aggregates. HFIP solution was evaporated under a ventilated fume hood by applying a light stream of N₂ gas. The HFIP film containing the A β peptide was either directly re-suspended in 100% DMSO and further diluted to 1% DMSO in a new buffer or stored dry at -20 °C until use. A β peptide fragments comprising of residues: 1-15, 1-22, 1-23, 1-24-NH₂, 1-25, 1-25-NH₂, 26-40, 24-40, (purity of \geq 97%) were purchased from GenicBio Ltd. (Shanghai, China). A β peptide concentrations were determined by UV absorbance using the peptide's molar extinction coefficient at 280nm.

2.2 Size Exclusion Chromatography of A β 42 ADDLs and transgenic mouse brain tissue extracts.

Size exclusion chromatography (SEC) fractionation was carried out using an ÄKTA Explorer FPLC (GE Healthcare) placed inside a cold (4 °C) chamber. A Superdex 200 10/300 GL column (GE Healthcare) was used and samples were eluted with either 25 mM ammonium acetate (pH 8.5) or a Superdex 75 10/300GL with 20mM Tris 20mM NaCl (pH7,5) (for aggregated A β 1-25), at a flow rate of 0.5 ml/min. Prior to injection, samples were centrifuged at 4°C 16,000 \times g for 20 min and 0.5ml of sample supernatant was injected onto the column. Aggregated A β 1-25 peptide was filtered using 0.22 μ m filter devices prior to injection to prevent from injecting any large, fibrillary aggregates. Peptide elution was detected by absorbance at 280 nm, 275nm and 215nm and 0.5 ml fraction volumes were

collected. Eluted fractions were either used immediately or aliquoted (50ul) and stored at -80°C. Where indicated, samples volumes were concentrated approximately 10x in a speed vacuum.

2.3 Matrix assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry (MALDI TOF/TOF).

Aliquots (2 µl) of samples were used for MALDI-TOF/TOF MS (ABI 4800 model, Applied Biosystems) measurements. Matrix solution of α -cyano-4-hydroxycinnamic acid (7 mg/ml in ACN/0.1% TFA (1:1, v/v)) was used for sample deposition. The sample (1 µl) was mixed with 1 µl of matrix solution and then 1 µl of this mixture was deposited in duplicates on the target plate and allowed to air dry. Samples were analyzed in reflectron positive mode.

2.4 Digestion of A β peptides.

Proteolytic digestion using LysN (2ng/ul) was performed overnight at 37 °C in 50 mM ammonium bicarbonate, pH 10 (LysN buffer). For in-gel digestions, coomassie stained gel bands were cut at the migration level of LMW A β oligomers (range: 6kDa - 14kDa) as revealed by their immunoreactive bands in WB. Gel bands were destained and dried in a speed vacuum prior to resuspension in Lys-N buffer containing 2ng/ul Lys-N protease followed by overnight digestion at 37°C. Following digestion, the solution was recovered and pooled with the peptides extracted from gels and concentrated by speed vacuum prior to LC-MS measurements. Immunoprecipitated samples (IP), were reduced and alkylated followed by in-solution digestion at 37°C using standard LysN buffer (50ul) and approximately 70ng of LysN. Dried samples were resuspended in 10% DMSO and 5% FA as described below, followed by LC-MS/MS or LC-SRM analysis.

2.5 Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

For high resolution LC-MS/MS analysis, peptides were resuspended in 2% ACN, 0.1% FA and separated by reversed-phase chromatography on a Dionex Ultimate 3000 RSLC nanoUPLC system connected in-line with an Orbitrap Elite (Thermo Fischer Scientific, Waltham, MA, USA). The instrument was operated in an information-dependent mode where peptide masses for light and heavy lysine (K) labelled fragments A β 16-23, A β 16-24-NH₂, A β 16-25 and A β 16-27 (purchased from Sigma Aldrich, Germany) were selected for collision-induced dissociation (CID) to generate tandem mass spectra using a normalized collision energy (CE) of 35. Samples were first captured on a homemade capillary pre-column (Magic C18; 3 μ m-200Å; 2 cm \times 100 μ m) prior to analytical separation. A 80-min biphasic gradient was run starting from 100% A solvent (2% acetonitrile, 0.1% formic acid) to 90% B solvent (100% acetonitrile, 0.1% formic acid) on capillary column (Nikkyo C18; 3 μ m-100 Å; 15 cm \times 75 μ m inner diameter at 250 nl/min).

2.6 Quantitation of A β peptide fragments using Selected Reaction Monitoring (SRM) mass spectrometry.

All samples were analysed on a TSQ-Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific). A 0.7-FWHM-resolution window for both Q1 and Q3 was set for parent- and product-ion isolation. Fragmentation of parent ions was performed in Q2 at 1.5 mTorr, using collision energies calculated with the Pinpoint software (v1.1). Cycle times of 0.5s-1s were used for SRM runs with a minimum dwell time of 20ms.

Parent-ion selection was set for Lys-N digested peptides on the positively-charged parent ions. CID fragmentation energies and the best transition selection were tested manually by infusion on the TSQ using the synthetic peptide standards listed below. Mouse or human

180 brain and CSF samples were extracted and prepared for digestion as outlined below.
181 Following overnight digestion at 37°C, samples were dried using a speed vacuum and stored
182 at –20°C until analyses were performed. For A β peptide quantitation studies, a mixture of
183 accurately quantified (by amino acid analysis) heavy isotope (lysine, K) labelled peptide
184 standards (Sigma Aldrich, Germany) comprising of residues: A β 16-23, A β 16-25, A β 16-27,
185 A β 28-38, A β 28-40, A β 28-42 and A β 28-43, were spiked into each tube after resuspension of
186 samples in the Lys-N digestion buffer. A β peptide fragments were initially resuspended in a
187 solution containing 20% DMSO & 10% formic acid (FA) and further diluted to 5% DMSO &
188 2.5% FA prior to injection and analysis by LC-SRM. This solution provided maximum long-
189 term stability of all peptide standards. Nano-LC-SRM parameters: Dried peptide aliquots
190 were resuspended in 20 μ l DMSO (10%) with 5% FA. This preparation provided peptide
191 solubility over two weeks without any significant changes in overall peptide recovery.
192 Following resuspension, samples were briefly sonicated (3min) and allowed to settle for 1h to
193 increase overall peptide solubility before analysis. Typically, 5 μ l of sample was loaded and
194 captured on a homemade capillary precolumn (C18; 3 μ m, 200 Å; 2 cm \times 250 μ m) before
195 analytical LC separation (ACQUITY UPLC, Waters). Samples were separated using a 60min
196 biphasic gradient starting from 100% solvent A (100% acetonitrile, 0.1% formic acid) to 90%
197 solvent B (100% acetonitrile, 0.1% formic acid) on a Nikkyo (Nikkyo Technology) nano-
198 column (C18; 3 μ m, 100 Å; 150mm length and 100 μ m inner diameter; flow of 0.5 μ l/min).
199 The gradient was followed by a wash for 8 min at 90% solvent B and column re-equilibration
200 for 15 min at 100%.

2.7 SDS-PAGE and Immunoblotting.

Dried amples were mixed using standard SDS Lämmli sample buffer, Novex SDS sample buffer and heated at 80°C for 5 min prior to loading onto gels. Three different commercially available gels were used in order to compare the migration behaviour of A β peptide fragments: Novex 16% Tris-Tricine gels, 1mm (Invitrogen), Biorad 10-20% Tricine gels (Biorad, Switzerland) and Novex Nupage 4-12% Bis-Tris gels, 1mm (Invitrogen), of which the latter type gels were used throughout the study. The following commercially available running buffers were used: Novex Nupage Mes-SDS buffer (Invitrogen), Novex Tricine-SDS buffer (Invitrogen) and Biorad Tricine buffer (Biorad, Switzerland). PAGE separated samples were electroblotted onto nitrocellulose (0.22 μ m) membranes using standard protocols as provided by the manufactures. Membranes were blocked for 1h at room temperature under constant rocking using Odyssey blocking buffer (Li-COR Biosciences, Bad Homburg, Germany) diluted 1:1 in PBS. Following blocking, membranes were incubated at 4 °C with constant rocking overnight using the primary rabbit polyclonal neo-epitope antibodies N-5ns, N-5s, N-4, N-3s, D-4s, or D-6ns (0.28-0.5 μ g /ml), or the commercially available mouse monoclonal antibodies 6E10 and 4G8 (0.5 μ g /ml) (Enzo, Life Sciences, Switzerland). Membranes were washed four times with PBS-Tween (PBS containing 0.01% Tween 20), followed by incubation with a goat anti-rabbit or anti-mouse secondary IgG antibody (highly cross-adsorbed) (dilution, 1:5000) conjugated to Alexa Fluor 680 or 800 and scanned in a LI-COR scanner at a wavelength of 700 nm and 800 nm respectively.

2.8 Dotblotting.

Typically, 1ul samples were spotted onto a nitrocellulose membrane, which corresponded to a total peptide load of 100ng (A β 1-40) and 50ng (A β 1-42) unless otherwise stated in the figures. Samples were left to dry for 15min followed by blocking of the membrane (30min) with LICOR buffer. Membrane strips were incubated with primary antibodies either for 2hrs at room temperature or overnight at 4°C on a shaker. Identical solutions, antibody concentrations and revelation procedures with secondary antibodies were used as described for immunoblotting above.

2.9 Generation of polyclonal antibodies.

Briefly, a hepta to deca peptide sequence corresponding to the target neo-epitope sequence of human A β peptide was conjugated to a KLH-linker and used for immunization of rabbits (e.g. A β 1-25: C+GG-VFFAEDVG-COOH). Antibodies (Table 1) were raised in rabbits against a peptide identical to C-terminal residues Gly25 (N-5ns & N-5s), Asp23 (N-3s), Val24-NH₂ (N-4), or N-terminal residues Val24 (D-4) and Ser26 (D-6) of human A β peptide. All polyclonal antibodies were affinity-purified against the target A β neo-epitope sequence using the carboxy- and amidated C-terminal form of the peptide sequence. Antibody specificity and affinity was validated using direct ELISA with surface immobilized (cross-linked to BSA) A β peptide sequences of normal and amidated C-terminus. All neo-epitope antibodies were prepared by Eurogentec SA, (Liege, Belgium).

2.10 In vitro A β peptide aggregation studies.

HFIP (1,1,1,3,3,3-Hexafluoro-2-propanol) (Sigma-Aldrich, Switzerland) dried A β peptide films were solubilized in DMSO and further diluted with PBS (or 30mM Tris, 150mM NaCl) to a final concentration of 0.1mg/ml for A β 1-40 and 0.05mg/ml for A β 1-42. Samples were incubated at 37°C and left for spontaneous aggregation during either 0-20 hrs (short-term), 1-5 days (intermediate) or 1-10 wk (long-term). Aggregated sample aliquots were drawn at different time points and either analysed immediately using MS and/or DB or snap frozen in liquid nitrogen and stored at -80°C. Typically, one microliter was spotted onto a nitrocellulose membrane for dotblotting (50ng to 100ng / spot) or mixed with alpha-cyano matrix for MALDI-TOF/TOF analysis. For heavy water (H₂¹⁸O, 97.0%, Cambridge Isotope laboratories Inc., MA, USA) peptide aggregation studies, HFIP dried A β peptide films were resuspended in anhydrous DMSO and diluted with H₂¹⁸O (containing Tris-NaCl 10mM/150mM) to a final DMSO concentration of $\leq 1\%$. The heavy H₂¹⁸O part of the final reaction solution was estimated at approximately $\geq 95\%$.

2.11 Immunoprecipitation of mouse and human brain tissue.

Mouse and human brain tissue samples were serially extracted using TBS, 2% SDS or formic acid (70-90%FA) as stated in the text. Briefly, tissue samples were homogenized (20 strokes on ice) in TBS and 5mM EDTA with protease inhibitor complex (Roche, Switzerland) using a Teflon homogenizer. Samples were then subjected to centrifugation (150,000g) during 45min and the supernatant was recovered as the TBS soluble fraction. Protein pellets were subjected to an additional extraction using either SDS (2%) or FA (70%) followed by centrifugation. Typically, pellets were extracted with FA overnight at 4°C (to minimize formylation adducts) followed by centrifugation. SDS fractions were diluted to

≤0.1% SDS final concentration and FA fractions were neutralized to pH 7.5 with 5M sodium hydroxide (NaOH) solution prior to IP. All samples were initially depleted of endogenous IgG's using a mixture of protein A&G agarose beads (Roche AG, Switzerland). Typically, 2-4ug/ml rabbit polyclonal antibody (N-3s, N-4 or N-5ns) or 3-5ug/ml of 6E10 or 4G8 mouse monoclonal was used for overnight IP under continues rotation (4rpm/min) at 4°C. Samples were eluted with 40% ACN /H₂O & 0.1% TFA and dried in a speed vacuum. IP'ed samples were either directly analysed by WB or MALDI-TOF/TOF or split and further digested overnight using LysN proteolysis for LC-MS/MS or SRM analysis as outlined above. Human CSF samples (500ul) were IP'ed with either N-5ns, or a mixture of the two commercial antibodies 6E10 and 4G8.

3. RESULTS

3.1 In vitro A β peptide aggregation is associated with autocleavage within the peptide's β -turn vicinity resulting in the generation of a typical peptide fragment signature.

We examined the aggregation behavior of synthetic A β 42 peptide using MS and observed that peptide aggregation is associated with a time dependent appearance of a typical peptide fragment signature *in vitro*. To rule out the possibility of artefactual peptide hydrolysis during sample preparation or MS analysis, we carried out aggregation studies using normal and heavy oxygen labelled water (H₂¹⁸O) to determine whether peptide cleavage is the result of aggregation-induced peptide hydrolysis.

MS analysis of A β 42 aggregation in both, normal and heavy (¹⁸O) labeled water produced identical MS spectra (Fig. 1A), revealing a highly reproducible A β peptide fragment signature following short term (t=12h) aggregation. Under heavy water labelled conditions, the majority of N-terminal fragment masses were shifted by 2 mass units as a result of a hydrolysis induced ¹⁸O atom incorporation at the newly formed C-terminus, as shown with one of the most abundant N-terminal fragment of residues A β 1-25 (Fig. 1A-G). A β 1-25 represents one typical truncated A β isoform from a selection of several large N-terminal fragments (Fig. 1B), where the monoisotopic peak was found to be shifted by 2.0012Da (0.5003Da for m/z = 734.3513 [M+4H]⁴⁺) as compared to the monoisotopic peak of normal (m/z= 733.8463 [M+4H]⁴⁺) (Fig. 1C, left) experimental conditions. No difference in mass shift was observed for the complementary C-terminal fragment of residues 26-42 indicating that hydrolysis-induced peptide cleavage resulted in stable ¹⁸O incorporation only at the neo-C-terminal residue Gly₂₅ (Fig. 1C, right).

We also observed substantial C-terminal amidation (CONH₂) within the same isotopic cluster of A β 1-25, where the mass shift between the unmodified monoisotopic peak (m/z=

733.8509 $[M+4H]^{4+}$) and the amidated monoisotopic peak ($m/z= 733.6037 [M+4H]^{4+}$) accounted for 0.988 Da (theoretical Δ mass = 0.984 Da) (Fig. 1C, right). The estimated relative abundance of C-terminal amidation of fragment A β 1-25-NH₂ has been estimated to account of approximately 20% of the normal, carboxy C-terminal population (data not shown).

Interestingly, A β 1-24 was found to be predominantly amidated (A β 1-24-NH₂) and only minor levels of the normal carboxy C-terminus were detected (data not shown). Overall, we observed that the gradual accumulation of the A β fragments of residues A β 1-23, A β 1-24-NH₂, A β 1-25 or A β 1-25-NH₂ reflect a typical fragment signature during the early phase of peptide aggregation because these large N-terminal fragments could be readily detected following short term aggregation (≤ 1 h) and typically preceded the accumulation of earlier reported A β isoforms such as A β 1-15 (Supplemental Fig.1). We also observed that some N-terminal fragments have increased aggregation propensities, as seen by the rapid formation of MS stable entities of dimeric and trimeric nature (Supplemental Fig.1 A&B) and that this in turn may affect LC-MS analysis. Therefore, to monitor the generation of these A β isoforms in a more reproducible manner, we processed samples using Lys-N proteolysis, which generates the proteolytic cleavage product of residues A β 16-25[20]. Lys-N digestion of A β peptides resulted in highly increased solution stabilities and MS detection (100 fold) of the proteolytic cleavage products as compared to non-digested A β 1-25 (Supplemental Fig. 1D). The change in overall charge distribution from multiply charged ions ($[M+2H]^{2+}$ to $[M+5H]^{5+}$) for A β 1-25 to a predominantly double charged ion ($[M+2H]^{2+}$) for the Lys-N fragment A β 16-25 would generally account for this significantly improved detection.

Quantitative MS analysis, using a spiked-in heavy lysine (K16) labelled surrogate peptide indicated, that the relative abundance of fragment A β 1-25 accounts for approximately 5% of full-length A β 42 (Fig. 1D). However, the absolute abundance of the here identified truncated

A β isoforms of A β 1-23, A β 1-24-NH₂ and A β 1-25 as well as the C-terminal amidated form would clearly exceed this level.

To further confirm a hydrolysis-induced peptide cleavage during *in vitro* aggregation, we applied MS/MS using collision-induced dissociation (CID) to identify the site of stable ¹⁸O incorporation. MS/MS analysis of the Lys-N digested A β 1-25 (16-25) fragment allowed for unambiguous identification of the ¹⁸O atom incorporation at Gly25 (Fig. 1E & F).

3.2 N-terminal A β peptide fragments are highly prone to oligomerization.

To determine the biophysical properties of truncated A β peptides, we investigated the aggregation properties of four large N-terminal fragments: A β 1-23, A β 1-24-NH₂, A β 1-25 and A β 1-25-NH₂. Following *in vitro* aggregation of the synthetic A β fragments (t=1wk) we observed significant changes in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) migration behavior as seen by the appearance of several low molecular weight (LMW) species in the range of 6kDa to 14kDa (Fig. 2A). High molecular weight (HMW) oligomers were observed in the 49kDa to 62kDa range and this oligomeric entity was found to be particularly characteristic for the amidated fragment A β 1-25-NH₂. Immunoblot analysis of some A β isoforms remains challenging using the conventional antibody 6E10, as seen for A β 1-23, which may result from conformation associated epitope masking [21], and therefore a decreased sensitivity for 6E10 when compared with 4G8.

MS detection of LMW oligomers was mainly achieved at the dimer and trimer level, such as shown with A β 1-25, which may highlight the metastable structure found with soluble oligomers during LC-MS analysis (Supplementary Fig. 1A & B). Moreover, prolonged aging of the truncated A β isoforms A β 1-25 or A β 1-25-NH₂ resulted in the generation of shorter N-terminal fragments such as the earlier reported fragments of residues A β 1-14 and A β 1-15

(Supplementary Fig. 1C & E). This observation was in agreement with prolonged A β 42 aggregation experiments (t=7d), showing a time dependent decrease in MS detection of A β 1-25 together with the gradual appearance of shorter fragments, such as A β 1-15 (Supplementary Fig. 2A).

Transmission electron microscopy (TEM) imaging indicated that N-terminal fragments have a high propensity to form soluble, oligomeric aggregates following long-term incubation. A β 1-25 preferentially formed homogenous spherical aggregates, which was less evident with the shorter N-terminal fragments of residues A β 1-23 and A β 1-24-NH₂ and only very few clusters of fibrils were observed with the amidated form A β 1-25-NH₂ (Fig. 2A and Supplemental Fig. 2F). We further employed SEC (in Tris-NaCl) of aggregated A β 1-25 and show that the LMW structures typically observed with these truncated A β isoforms are true observations and can therefore exclude a SDS-PAGE induced migration artefact (Fig. 2B). SEC fractionation resulted in a clear separation of two A β structures centered at the migration level of ≤ 6 kDa (fraction: 12ml) and ≤ 3 kDa (fraction: 15ml) and MS analysis of fraction volume 12ml revealed the presence of stable A β 1-25 dimers and trimers, whereas monomers were mainly detected in fraction volume 15ml (data not shown). Because A β 1-25 dimers (5.8kDa) and trimers (8.7kDa) were highly enriched in SEC fractions corresponding to a MW standard of ≤ 14 kDa, we speculated that LMW A β 1-25 structures of may form part of larger A β entities, which dissociate during SDS-PAGE analysis.

3.3 Monitoring A β peptide aggregation using neo-epitope antibodies which specifically target truncated A β isoforms.

To create a simple analytical tool for the detection of site specific autocleavage during aggregation we set out to develop an antibody enabled proof-of-principle tool for monitoring A β cleavage. We therefore developed a panel of rabbit polyclonal antibodies (Table 1) with high binding specificity for N-terminal fragments of residues A β 1-23 (N-3s), A β 1-24-NH₂ (N-4) and A β 1-25 (N-5s & N-5ns) (Fig. 3 & Supplemental Fig 3A), as well as for two complementary C-terminal fragments: A β 24-42 (D-4s) and A β 26-42 (D-6ns) (Supplementary Fig. 3B).

Epitope binding was tested by dotblot (DB) using a repertoire of synthetic A β peptide standards (1 μ g/spot) of different sequences or C-terminal endings and binding specificity was compared with the conventional antibodies 6E10 and 4G8. None of the neo-epitope antibodies detected full-length A β 40 peptide, which highlights their unique binding specificity for cleaved A β isoforms. Overall, 4G8 (17-24) showed higher specificity with respect to its binding epitope as compared to 6E10 (1-16) because detection of fragment A β 1-15 was significantly reduced with 6E10, which is an observation reported before [22].

N-5s showed high specificity for the C-terminal Gly25 residue and N-3s selectively detected fragments ending with C-terminal residue Asp23. The N-5ns binding epitope was found to include residues spanning the neo-C-terminal region of residues 23-25, with preferential binding properties for C-terminal Gly25 (Supplemental Fig. 3A) and binding was significantly decreased or absent with the shorter fragments of A β 1-22 and A β 1-15 respectively. Similarly, antibody N-4 revealed high binding specificity for the amidated C-terminal form of Val24 (A β 1-24-NH₂) (Fig. 3B). Because of the analytical limitations observed with DB (native conditions) we further compared the neo-epitope antibody selectivity to 6E10 using immunoprecipitation (IP) of an A β fragment mixture along with

full-length A β 40 (Fig. 3C) or in presence of a complex human proteome matrix (brain tissue extract) (Supplementary Fig. 4C & D). All neo-epitope antibodies showed high selectivity for their target fragments, whereas IP with 6E10 resulted in a pull-down of all A β fragments together with full-length A β 40.

The application of neo-epitope antibody N-5ns was further tested using ELISA and WB. At working concentrations of $\leq 0.3\mu\text{g/ml}$, N-5ns showed significantly lower affinity for the C-terminal amidated form A β 1-25-NH₂ (Fig. 3D) and WB revealed high selectivity for the target fragments (Fig. 3E&F). In summary we can conclude that, by using a combination of different conventional techniques, we were able to show that the above mentioned neo-epitope antibodies have unique binding properties for truncated A β isoforms and may serve as complementary tools for the analysis of biological samples.

3.4 A β peptide cleavage is highly associated with changes in peptide secondary structure.

In order to understand the mechanisms associated with A β peptide cleavage during aggregation we investigated peptide cleavage by stabilizing A β secondary structure in different solutions. In PBS solution (or 40mM Tris: data not shown), A β 40 showed a predominantly unordered structure with considerable decrease in signal amplitude over the time course of incubation, whereas incubation in 20% TFE solution induced a stable α -helical structure during five days of incubation (Fig. 4A). Significant A β cleavage was observed in PBS or Tris solutions, whereas cleavage was strongly attenuated in TFE (Fig. 4 B & C), indicating that A β cleavage is highly associated with changes of the peptide's secondary structure as a result of peptide aggregation. This observation was also true for A β 42 (data not shown).

Having established neo-epitope antibody specificity, we then selected and employed N-5ns to monitor *in vitro* A β 42 aggregation using DB and MS. We observed a robust increase in N-5ns DB signal shortly after peptide incubation indicating a nearly instantaneous accumulation of diagnostic A β fragments and TEM imaging of the peptide morphology indicated that there was a correlation between the N-5ns signal saturation by DB and the presence of typical oligomeric and protofibrillar aggregates (Fig. 4D).

We further studied the effects of different experimental conditions in A β aggregation. Earlier findings provided strong evidence that the protein cross-linking activity of tissue transglutaminase (tTGase) plays an important role in AD pathogenesis [23] [24]. We therefore sought to investigate if tTGase induced A β aggregation mirrors the accumulation of A β fragment fingerprints. A β 40 aggregation increased significantly in the presence of tTGase and was accompanied by the formation of low amounts of intra- and intermolecular cross-links (data not shown) as reported previously [25] [18]. This change in aggregation behavior could be detected by DB (Fig. 4E) and IP-MS analysis confirmed the presence of the N-5ns target fragments A β 1-23 and A β 1-25 in samples subjected to different aggregation conditions (Fig. 4F).

Since we were able to show that several peptide cleavage sites represent a typical hallmark of early aggregation, we argued that the sum of several cleavage products would increase overall DB screening sensitivity and showed, that the combination of several neo-epitope antibodies indeed increased detection sensitivity during *in vitro* peptide aggregation (Supplementary Fig. 4A). We further reasoned that neo-epitope antibody-based monitoring of A β cleavage, prior to the accumulation of β -sheet enriched fibrils, would provide valuable information for future screening natural inhibitory compounds (Supplementary Fig. 4B) [26] [27] [28] [29]. This novel screening concept would be complementary to the typically employed Thioflavin-T fluorescence measurements, since N-5ns would allow identification

of aggregation inhibitory compounds targeting an oligomerization fate [21] [30] rather than advanced fibrillation.

3.5 A β fragments form stable complexes with soluble oligomers.

To better understand the biophysical properties of truncated A β in peptide oligomerization, we investigated the aggregation kinetics of amyloid-derived diffusible ligands (ADDL's) using a combination of SEC, DB, WB and MS. Following 20hrs of A β 42 oligomerization, we observed a substantial increase in N-5ns signal using DB (Fig. 5A) and this positive signal was associated with the presence of a typical oligomeric morphology (data not shown). Following *in vitro* aggregation, the ADDL preparation was centrifuged in order to precipitate large insoluble aggregates (pellet) and the supernatant containing soluble A β species was subjected to SEC fractionation. To identify SEC fractions containing truncated A β isoforms, 50ul fractions were dried by speed vacuum and re-suspended in 5ul of PBS of which 1ul was spotted in duplicates onto a nitrocellulose membrane and probed by DB using N-5ns and 6E10 respectively. SEC fraction probing with N-5ns allowed identification of a substantial amount of truncated A β within the oligomeric fractions (8ml - 12ml) (Fig.5A). DB probing with 6E10 and N-5ns of the monomeric fraction (18ml) indicated that this fraction contained a mixture of both, A β 42 monomers as well as truncated A β isoforms and WB analysis of these SEC fractions was in line with the findings from DB (Fig. 5B). Moreover, WB also revealed the presence of HMW oligomers centered at the 49kDa to 62kDa range. The presence of low amounts of this HMW entity observed in the monomeric fraction may indicate that truncated A β fragments may favor the formation of these HMW structures as a result of sample concentration by speed-vacuum.

To further validate the N-5ns positive signals found by DB and WB, we analyzed oligomeric fractions using IP-MS. MS analysis of the “crude” fraction volume 8ml revealed that this fraction contained fragments A β 1-23, A β 1-24-NH₂, A β 1-25 and A β 1-25-NH₂ along with some shorter fragments of A β 1-17 to A β 1-22 as well as full-length A β 42 (Fig. 5C; top). Similarly, MS analysis of LMW A β species extracted from SDS-PAGE gel bands (\leq 14kDa) confirmed the presence of different truncated A β isoforms as well as their complementary C-terminal fragments (Supplementary Fig.5).

IP with N-4 resulted in a specific pull-down of fragment A β 1-24-NH₂ (Fig. 5C; center) along with A β 42 and N-5ns allowed a specific recovery of fragments A β 1-25 but not A β 1-25-NH₂, as well as trace amounts of A β 1-23 together with A β 42 (Fig. 5C; bottom).

To further elucidate the importance of A β 1-25 in oligomers, we analyzed two oligomeric (11.5ml and 14ml) and a monomeric fraction (18ml) using IP-MS (N-5ns) (Fig. 5D) and could identify A β 1-25 in both, oligomeric and monomeric fractions. The relative abundance of this particular fragment varied considerably, which was in line with our DB and WB analysis. IP with N-5ns also resulted in a pull-down of A β 42 indicating that oligomeric, as well as monomeric fractions contained metastable complexes of fragment A β 1-25 and A β 42, which may dissociate to a generally monomeric level during SDS-PAGE analysis. This data collectively confirms the above reported observation that truncated A β can form stable entities with soluble oligomers.

To provide quantitative values for A β 1-25 and A β 42 we used IP combined with LC-SRM analysis. SEC fractions were split and one part was denatured with 70% FA over-night at 4°C to allow gradual dissociation of large oligomeric species. We reasoned that this approach would reduce the overall recovery (pull-down) of full-length A β 42 stably bound to the surface of large oligomers and therefore allow more accurate quantitation of the A β 1-25 target fragment *per se*. SRM quantitation confirmed the significantly lower levels of A β 1-25

(2-3pg/ul) (Fig. 5E) found in fraction volume 11.5ml as compared to the monomeric (18ml) fraction (21-23pg/ul), which is also in line with our WB analysis. Moreover, we found that FA dissociation of oligomers significantly reduced (40x) the amount of full-length A β 42 stably bound to fragment A β 1-25. IP-MS of the monomeric fraction, using a combination of three different neo-epitope antibodies, revealed the presence of three truncated isoforms: A β 1-23, A β 1-24-NH₂ and A β 1-25 together with A β 42 (Fig. 5F).

Because our initial mock IP's of freshly prepared A β fragment mixtures did not pull-down full-length A β 40 or A β 42, we sought to provide direct evidence that detection of A β 42 within the monomeric fraction is the result of a collective pull-down due to stable interaction of truncated A β and A β 42. For this purpose, we denatured the first IP sample over-night using 90% FA, followed by a second IP using the same antibody cocktail. MS analysis of the sequential IP (2nd) confirmed our assumption, because A β 42 was no longer detected following FA treatment (Fig. 5G).

MS analysis of the insoluble, pellet fraction (from ADDL prep.) indicated that large insoluble aggregates also consist of heterogenic entities rich in N-terminal as well as C-terminal truncated A β isoforms together with full-length A β 42 (data not shown). This observation was further corroborated by the finding that IP with antibody N-5s or N-5ns of 7M guanidine hydrochloride (GHC1) denatured A β 42 fibrils, resulted in a specific recovery of A β 1-25 together with trace amounts of A β 42 (Supplementary Fig. 6A & B).

3.6 N-terminal A β peptide fragments are present in the amyloid deposits in the human AD brain.

Immunohistochemical staining (both chromogenic and fluorescent) of the frontal cortical tissue of human sporadic AD subjects (N = 6 AD cases and 3 age-matched non-demented controls) using N-5ns resulted in robust labeling of thioflavin-S-positive amyloid plaques and cerebral amyloid angiopathy (CAA) (Fig. 6A & B). Thioflavin-S-positive intraneuronal tau tangles were not labelled with N-5ns (Fig. 6B) and no non-specific labeling was detected in the control non-AD brains (Fig. 6C). Because one of the early and important effects of oligomeric A β in AD is the binding of A β to synapses and the resultant synaptic dysfunction and loss [31], we examined the presence of A β neo-epitopes in synapses. Tissue from human subjects was prepared for high-resolution array tomography [32], allowing accurate detection of individual synapses. As seen with the immunostaining of paraffin sections, N-5ns labeled amyloid deposits that were positive for thioflavin-S and 6E10 (Fig. 6D). Both dense-core and diffuse plaques were immuno-positive for N-5ns. We also observed staining of N-5ns at individual pre- and postsynaptic puncta in the region of plaques, indicating that this A β fragment may be important in synapse degeneration (Fig. 6E).

3.7 Analysis of human AD brain and transgenic mice brain tissue highlights the significant abundance of A β cleavage in AD.

In order to further corroborate our *in vitro* observation on A β cleavage, we analyzed human post-mortem brain tissue samples from human controls and AD subjects. We employed IP-MS and WB analysis of TBS and FA tissue extractions to investigate presence of A β truncation using the two conventional antibodies 6E10 and 4G8 and compared A β peptide recovery with neo-epitope antibodies. IP with 6E10&4G8 resulted in the detection

of a band centered at the typical migration level of A β monomers (>3kDa) (Fig. 7A, top blot: AD#1 lane: 1, AD#2 lane: 4), whereas IP with neo-epitope antibodies revealed a band centered at the migration level of \leq 6kDa for one AD subject (Fig. 7A top blot, lane: 5). Interestingly, re-probing the membrane with a neo-epitope antibody cocktail revealed a band centered at the migration level of \geq 3kDa (Fig. 7A bottom blot: AD#1 lane: 2, AD#2 lane: 5), indicating that both AD samples contained truncated A β isoforms, whereas 4G8 failed to show similar specificity at this migration level, which is an observation also made with the synthetic A β fragments (Fig. 3).

We further analyzed the same brain samples by MALDI-TOF/TOF MS. IP-MS (6E10&4G8) analysis of the AD#2 brain tissue extract (FA) resulted in a pull-down of large A β fragments A β 36, A β 37, A β 38 and A β 40 together with several N-terminal fragments A β 1-13-NH₂, A β 1-13, A β 1-20, A β 1-22, A β 1-23, A β 2-24-NH₂, A β 1-24-NH₂ and A β 1-25 (Fig. 7B). IP-MS analysis of the same AD brain, using a combination of three neo-epitope antibodies resulted in a specific pull-down of the target fragments (Fig. 7C). The same samples were also subjected to LC-MS/MS (Orbitrap) analysis for high resolution peptide mass confirmation (Fig. 7D & E).

Moreover show that, IP with N-5ns enables a specific recovery of fragment A β 1-25 from Tg2576, APP/PS1 (Supplementary Fig. 6C & D) and 5xFAD (data not shown) transgenic mice brains. The use of SDS (Supplemental Fig. 7), TBS or FA (Supplementary Fig. 8A & B) extraction protocols all resulted in similar truncated A β recovery, however, the levels of A β 1-25 were found to be significantly increased in FA extracts as compared to TBS soluble fractions (Supplementary Fig. 8B). LC-SRM analysis of TBS and FA brain extracts from human controls indicated that the levels of cleaved A β isoforms are significantly reduced or below the limit of detection (data not shown).

3.8 Human CSF analysis reflects the accumulation of N-terminal fragments in AD brain.

We first measured levels of A β 42 and total Tau (T-Tau) in AD patients and non-demented (ND) controls using ELISA and observed significantly ($p < 0.001$) decreased levels of A β 42 as well as increased T-Tau levels ($p < 0.001$) (Supplementary Fig. 8C & D) in AD patients as compared to ND controls, which is in line with earlier reported measurements of human CSF samples [11, 33]. We were further interested in identifying A β cleave by applying quantitative IP-SRM (N-5ns) to measure A β 1-25 levels in CSF and could clearly confirm the presence of A β 1-25 in both; AD patients and age matched controls subjects (Supplementary Fig. 8E). A large inter-subject variability of A β 1-25 levels was generally observed in AD patients ($n=16$) but the measured levels failed to show a statistical significant difference ($p > 0.05$) when compared to control subjects ($n=14$) (Supplementary Table II).

4. DISCUSSION

The presence of particular A β peptide fragments *in vitro* [34, 35] and *in vivo* [36, 37] has been reported before and the accumulation of some A β isoforms is thought to be associated with a putative enzymatic activity [38-40]. Generally, it seems unlikely that either proteinases or exopeptidases are responsible for the generation of truncated A β isoforms because *in vitro* peptide cleavage still occurs in the presence of a metalloprotease inhibitor [35] or bacteriostatic agents (data not shown). Overall, the exact mechanism associated with the putative concerted enzymatic cleavage of A β still remains unclear. Regardless of proteinase activity, earlier reports show that *in vitro* A β 40 aggregation resulted in peptide cleavage at residue Asp23 [41] and that the increased aggregation observed for A β 40 in the presence of

tTGase was accompanied with significant cleavage at residues Glu22 and Gly25 respectively [18]. Moreover, it has been suggested that A β 25-35 as well as full-length A β 40 mediated toxicity may result from a peptide cleavage induced radicalisation of cell membranes, and that prolonged incubation resulted in significant A β cleavage at the Gly25-Ser26 bond [42]. The here reported occurrence of cleavage at residue Gly25 is of particular interest, because A β 1-25 was earlier identified as a sphingolipid binding domain motif, which can be rapidly internalized by neuronal cells [43].

We report here new, additional A β fragments and were able to demonstrate that the gradual appearance of fragments A β 1-23, A β 1-24-NH₂ A β 1-25 and its amidated form A β 1-25NH₂ can be associated with the early events of A β aggregation, because MS detection of the aforementioned N-terminal fragments precedes the accumulation of shorter A β isoforms (i.e A β 1-15).

To our knowledge, the presence of substantial C-terminal amidation *in vitro* and *in vivo* has not been reported before, suggesting that A β cleavage is the result of at least two distinct molecular mechanisms. More importantly, we show that A β cleavage is strongly attenuated when stabilizing the peptide in an α -helical structure. This suggests that the transition from an unstructured, random-coil conformation to a β -sheet ordered structure triggers the cleavage cascade typically observed during peptide aggregation. Moreover, the time-dependent increase in A β 1-15 abundance may indicate that shorter N-terminal fragments represent cleavage products associated with a more advanced phase of aggregation, which is corroborated by the observation that long-term incubation of the here described fragment of A β 1-25, or its more amyloidogenic form A β 1-25-NH₂, give rise to shorter A β isoforms. Generally, the detection of a particular A β fragment signature in different AD transgenic models as well as human AD brains; collectively highlight the need for further understanding

the presence of A β fragment signatures in AD. Therefore, the exact molecular mechanism leading to site specific hydrolysis of A β remains to be elucidated in future studies.

Soluble A β oligomers play a central role in AD pathogenesis, with dimers [44] and dodecamers (A β * 56) having attracted most of the scientific attention in the past. We report here that IP of soluble A β 40 or A β 42 oligomers resulted in a specific recovery of A β assemblies with a gel migration range of ≥ 6 kDa, and that similar A β assemblies could be detected in TBS and FA lysates from human AD brains. Given by our findings, it is conceivable that A β assemblies of putative dimeric or trimeric nature consist of a mixture of truncated and full-length A β , which may form metastable complexes with HMW structures, which is partly in agreement with earlier reports [19].

The detection of A β 1-25 in CSF samples from human controls may suggest a significant abundance of A β oligomers present in control subjects, which is in line with earlier reports [45]. Interestingly, Holtta et al. (2013) showed that CSF oligomers were significantly increased in patients with mild and moderate dementia when compared to controls, whereas no significant difference was found in patients with severe dementia [12]. The here observed lack of statistical significance in A β 1-25 levels in AD patients may result from the relatively small sample size and hence statistical power. It is also conceivable that CSF sample freeze-thaw cycles together with other, earlier reported confounding factors[46, 47] may have collectively contributed to a rapid *ex vivo* A β aggregation in these samples.

In conclusion, we argue that neo-epitope antibodies would serve as appealing capture antibodies for future ELISA developments because we were able to show that the here described A β fragments can self-propagate to dimers and trimers or form stable entities with large oligomers. However, measuring changes in levels of truncated A β isoforms merits additional, future analytical improvements. We believe that monitoring pathological changes in A β levels in human CSF [39] or plasma [48] requires the use of multiplexed approaches,

where truncated A β isoforms together with several earlier reported A β fragments [49] as well as pathologically relevant post-translational modifications, such as pyroglutamate modified A β [16], should be monitored simultaneously and longitudinally in human biofluids.

RESEARCH IN CONTEXT

Systematic Review: A β dimers, trimers and dodecamers have received the most scientific attention in the past, because these entities have been suggested to form the building blocks of larger neurotoxic assemblies. However, the key molecular triggers associated with early A β oligomerization are poorly understood and to date, the exact molecular structure of LMW oligomers still remains a conundrum.

Interpretation: We have identified here new truncated A β isoforms with high aggregation propensities, which may serve as seeding units during early peptide aggregation. We provide analytical evidence that these truncated A β isoforms are highly abundant in A β oligomers.

Future directions: We plan to further study the generation of truncated A β isoforms as well as their significance to the pre-symptomatic accumulation of neurotoxic oligomers. The use typical A β peptide fragment fingerprints for a pre-symptomatic diagnosis of subjects suffering from MCI or other forms of dementia will be of particular interest. Furthermore, we are interested in studying the structural properties and aggregation fate of the here described truncated A β isoforms. This will help to identify and understand the structure homology found in A β oligomers which in turn may improve the future development of oligomer specific antibodies.

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SUPPLEMENTARY DATA

Supplementary figures 1-8 and tables I & II.

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